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EXHIBIT A

The Grb7 Family of Signalling Proteins

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ABSTRACT. The Grb7 family is a rapidly emerging group of Src homology (SH)2 domain-containing signalling proteins that currently contains three members, Grb7, 10 and 14. These proteins possess a conserved multidomain structure, including a central region exhibiting significant homology to the *Caenorhabditis elegans* protein Mig10. Differences in tissue expression and SH2 binding selectivity suggest that these adaptor proteins function in a tissue-specific manner to link specific receptor tyrosine kinases (RTKs) and other tyrosine phosphorylated proteins to as yet uncharacterised downstream effectors. Interestingly, Grb7 proteins exhibit differential expression amongst a variety of human cancers and cancer cell lines. Consequently, these proteins not only are likely to perform a fundamental signalling role, but may also modulate RTK signalling in cancer cells. CELL SIGNAL 10;9:613–618, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Grb7, Mig 10, SH2 domain, Tyrosine kinase

NTRODUCT ON

This decade, the characterisation of molecular interactions mediated by a variety of non-catalytic protein modules has provided a new conceptual framework for signal transduction events initiated by tyrosine kinases. Many proteins that function downstream of tyrosine kinases possess Src homology 2 (SH2) domains, conserved regions of approximately 100 amino acids that bind to short peptide sequences containing phosphotyrosine [1, 2]. These domains mediate interaction with autophosphorylated receptor tyrosine kinases and other tyrosine phosphorylated proteins, with the specificity of interaction being determined both by the amino acids flanking the phosphotyrosine and by the SH2 domain residues that interact with these sites. These domains function in concert with other modules to direct the formation of signalling complexes. For example, SH3 and WW domains target proline-rich peptide ligands with a PXXP and XPPXY core sequence, respectively, whereas both protein and phospholipid ligands have been described for pleckstrin homology (PH) domains [1–5].

Underscoring the importance of protein–protein interaction is the existence of a large subgroup of SH2 domain-containing proteins that lack enzymatic activity and contain only non-catalytic modules. These proteins function as adaptors, linking separate catalytic subunits to tyrosine phosphorylated proteins. Examples of this class are growth factor receptor bound (Grb) 2 and the p85 subunit of phosphatidylinositol 3-kinase [2, 6–8]. The former couples tyrosine kinases to Ras signalling through recruitment of the Ras GDP–GTP exchange factor Sos, whereas the latter binds a separate p110 catalytic moiety. Currently, numerous examples of the adaptor subgroup exist [2, 8]. However, the signalling mechanism and function of many of these proteins remain unresolved. This review focuses on a rapidly emerging family of adaptors, the Grb7 family, which, owing to their conserved structure, receptor recruitment profile and expression pattern in human cancers, are likely to perform a fundamental role in tyrosine kinase signalling.

STRUCTURE OF THE Grb7 FAM LY

Each member of the Grb7 family was originally cloned by the CORT (cloning of receptor targets) technique, in which cDNA expression libraries are screened with the tyrosine phosphorylated C terminus of the epidermal growth factor receptor (EGFR) [9]. According to a recently adopted nomenclature system for this family, the species of origin is now indicated at the beginning of the name and particular splice variants are designated by greek letters. Consequently, the proteins originally identified are now classified as mGrb7 [10], mGrb10α [11] and hGrb14 [12]. The proteins exhibit a conserved molecular architecture, consisting of an N-terminal region harbouring a conserved proline-

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Abbreviations: βARK–β-adrenergic receptor kinase; CAN–canal-associated neurone; CORT–cloning of receptor targets; EGFR–epidermal growth factor receptor; GM–Grb-Mig; Grb–growth factor receptor bound; HSN–hermaphrodite-specific neurone; IGF-IR–insulin-like growth factor I receptor; IR–insulin receptor; IRS–insulin receptor substrate; PCR–polymerase chain reaction; PH–pleckstrin homology; PTB–phosphotyrosine-binding; RTK–receptor tyrosine kinase; SH–Src homology.

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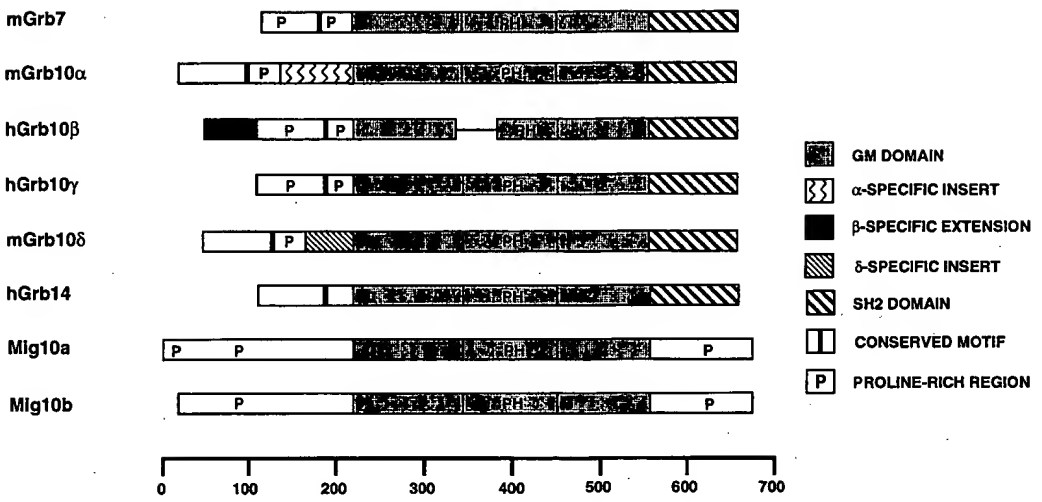

F GURE 1. Structure of Grb7 family proteins and their relationship to C. *elegans* Mig10. The proteins have been aligned to emphasise the conservation of the central Grb-Mig domain. The solid black bar indicates the position of the conserved proline-rich motif in the Grb7 family. The δ-specific insert is the same as the α-insert except for the deletion of 25 amino acids (see text). The numbers on the scale refer to distances in amino acids.

rich motif, a central region that exhibits homology to the C. *elegans* protein Mig10 and contains a PH domain and a C-terminal SH2 domain (Fig. 1).

The N termini of the Grb7 family exhibit the least similarity overall, but all contain a highly conserved proline-rich motif PS/AIPNPFPEL that is likely to be a binding site for a common regulatory or effector molecule. Also of note is that the N termini of Grb7 and Grb10 contain other proline-rich regions. The central or Grb and Mig (GM) region [13] spans approximately 320 amino acids and exhibits approximately 50% amino acid identity overall amongst the Grb7 family and approximately 30% identity between these proteins and Mig10. A key feature of this region is the central PH domain, although a highly conserved stretch of approximately 90 amino acids amino-terminal to the PH domain may constitute an additional functional module. Finally, the SH2 domains of the Grb7 family are approximately 70% identical, although they display distinct binding selectivities determined by amino acid substitutions at key positions [14].

Soon after the initial characterisation of mGrb10α, several laboratories searching for targets of the insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) receptor tyrosine kinase (RTK) by using yeast two-hybrid screens isolated cDNAs representing alternatively spliced *GRB10* homologues (Fig. 1). Liu and Roth [15] identified a variant in which the encoded protein (hGrb10β) exhibits a 55 amino acid extension at the N terminus and a 46 amino acid deletion that removes the N-terminal part of the PH domain. hGrb10γ [16, 17] lacks the 80 amino acid insertion between the conserved proline-rich motif and PH domain characteristic of mGrb10α and is therefore co-linear with mGrb7 and hGrb14, whereas, in mGrb10δ [18], only 25 amino acids of this insertion are deleted. The deletions and insertions found in these variants are likely to alter the activity of important functional domains, and so characterisation of isoform-specific properties is currently a priority. Furthermore, although in each case reverse-transcription PCR analyses confirmed the existence of mRNAs encoding these variants, the relative expression levels of the encoded proteins currently remain unknown.

As discussed earlier, the Grb7 family exhibit significant homology to the C. *elegans* protein Mig10. Two Mig10 variants that possess different N termini owing to the use of alternative 5' exons were recently characterised [13] (Fig. 1). Mig10a is slightly larger and exhibits two proline-rich regions in the N terminus, compared with only one such region in Mig10b. Both proteins possess common GM domains and C termini. Although the GM domains, by definition, are similar to those of the Grb7 family, it is important to note that the proline-rich regions are not homologous to the conserved motif characteristic of Grb7 proteins, and there is no SH2 domain. The potential functional relationship between Mig10 and Grb7 proteins is discussed later in this review.

EXPRESS ON OF THE GRB7 FAM LY N NORMAL T SSUES AND CANCERS

Northern analysis has revealed that the *GRB7* family displays overlapping but distinct expression profiles amongst a wide range of human tissues. *GRB7* is highly expressed in pancreas, kidney, placenta, prostate and small intestine [16]; *GRB10* in pancreas, skeletal muscle, brain and heart [16]; and *GRB14* in pancreas, kidney, skeletal muscle, liver, heart, ovary and testis [12]. Of note is that they are all expressed at very low or undetectable levels in thymus and peripheral-blood leukocytes.

The first indication that the *GRB7* family may exhibit differential expression amongst certain cancer types came

from the chromosomal localisation of the *GRB7* gene, which mapped to a mouse chromosome 11 locus syntenic to a region of human chromosome 17q commonly amplified in breast cancer [19]. This region also contains the gene encoding the ErbB2 RTK, which is over-expressed in approximately 20% of primary breast cancers owing to amplification events or transcriptional up-regulation or both [20]. Stein *et al.* [19] found that *GRB7* was tightly co-amplified with *ERBB2* in human breast cancer cell lines and that there was a strong correlation between ErbB2 and Grb7 over-expression in primary breast cancer specimens. Furthermore, Grb7 co-immunoprecipitated with tyrosine-phosphorylated ErbB2 from lysates of breast cancer cell lines expressing these proteins, suggesting that the co-amplification of these two genes up-regulates an ErbB2 signalling pathway. More recently, analysis of the *ERBB2* amplicon in other cancer types revealed that *GRB7* is also co-amplified with *ERBB2* in certain human gastric and esophageal cancer cell lines [21]. Furthermore, in a study of esophageal cancers, over-expression of *GRB7* relative to adjacent normal mucosa was detected in 44% of the specimens, and co-expression of *GRB7* with *EGFR* or *ERBB2* was significantly related to extramucosal tumour invasion [22].

It is now evident that the *ERBB* and *GRB7* family co-segregated during evolution. Thus *GRB10* localises to a region of mouse chromosome 11 [11] and human chromosome 7 [23] close to *EGFR* (*ERBB1*), and *GRB14* localises to the same arm of human chromosome 2 as *ERBB4* [24]. Interestingly, fluorescence in situ hybridization analysis with the use of a *GRB14* probe also suggested the existence of an additional related gene at 12q13, close to *ERBB3*. Although *EGFR* is amplified in certain human cancers—for example, glioblastoma [25]—it is currently not known whether *GRB10* undergoes co-amplification. However, although there are no reported cases of *ERBB4* amplification, *GRB14* is over-expressed in a subset of human and prostate cancer cell lines [12]. Consequently, differential expression in particular human cancers may be a more general feature of this family and may modulate RTK signalling during tumour progression.

S GNALL NG BY THE Grb7 FAM LY

SH2 Domain-Dependent nteractions

As described earlier, coupling between a RTK and a Grb7 family member *in vivo* was first described for ErbB2 and Grb7 [19]. In this study, Far-Western analysis with the use of Grb7-derived fusion proteins revealed that the SH2 domain was sufficient for interaction. Interestingly, this work also revealed that Grb7 associated through its SH2 domain with the adaptor protein Shc after growth factor treatment. It was previously well established that Shc becomes tyrosine phosphorylated after activation of a variety of growth factor and cytokine receptors and then binds the SH2 domain of Grb2, thus providing an alternative route to Ras activation [26]. Consequently, the recruitment of both Grb2 and Grb7 by Shc was the first suggestion that their SH2 specificity may overlap.

Subsequently, the interaction site on ErbB2 was defined [14], and other proteins that target the SH2 domain of Grb7 were identified, including the RTKs Ret [27] and the platelet-derived growth factor (PDGF) β-receptor [28] and the protein tyrosine phosphatase (PTP) SH-PTP2 [29]. In the Ret study, the Grb7 binding site was not mapped, but, in the remaining studies, the Grb7 binding site corresponded to a consensus motif of pYXN, which is also selected by the Grb2 SH2 [30]. Similarly, the Grb7 binding site(s) on Shc is likely to fit this consensus [31, 32]. Certain structural features common to the Grb2 and Grb7 SH2 domains may contribute to this similar binding selectivity [14]. However, although the binding selectivities overlap, they are not identical, and other residues in the binding site can act as selectivity determinants. For example, in a recent study, Y1180 and Y1243 of ErbB3 were found to target Grb7 and not Grb2, despite exhibiting pYXN motifs (manuscript submitted). An important functional consequence of competition between Grb2 and Grb7 for shared binding sites may be the modulation of Ras signalling, although this will depend on their relative affinities for particular sites and the nature of the effector pathway coupled to Grb7.

The use of yeast two-hybrid technology by several laboratories allowed the identification of Grb10 as a potential target of the IR and IGF-IR [15–18, 33–35]. The interaction between Grb10 and these receptors is of particular interest because it is independent of the docking proteins insulin receptor substrate 1 (IRS-1) and IRS-2, which recruit other SH2 domain-containing signalling proteins taking part in IR and IGF-IR signalling cascades [36]. Three groups were able to confirm association between Grb10 and the IR *in vivo* by co-immunoprecipitation analyses by using cell lines transfected with IR constructs and exhibiting endogenous Grb10 expression [16, 18, 34]. However, whether Grb10 associates with the IGF-IR in living cells is more controversial. Morrione *et al.* [35] reported co-immunoprecipitation of these two proteins; but, in a comparative study by the Smith laboratory using IGF-IR^{-}/− fibroblasts transfected with either the IR or the IGF-IR, only strong association between the IR and Grb10 could be detected [18].

Conflicting results have also been presented for delineation of the Grb10-binding site on the IR. Initially, it was mapped to the C-terminal autophosphorylation site Y1322 [34], but subsequent studies concluded that this site was not necessary for interaction [16, 17]. Indeed, these results indicated that Grb10 binds to sites within more than one of the IR subdomains (juxtamembrane, kinase and C terminus). Phosphopeptide competition analysis suggested that sites located in the former two regions were more likely to contribute to binding [16]. Furthermore, although the SH2 domain was sufficient for Grb10–IR interaction, a further region also may contribute to binding, because the full-length protein bound more strongly than the SH2 domain alone [16]. In the case of the IGF-IR, use of receptor deletion mu-

tants in the two-hybrid system implicated a C-terminal subdomain in Grb10 binding [35].

Two-hybrid cloning strategies have identified two other RTKs that recruit Grb10, Ret [37] and the Eph family receptor ELK (now termed EphB1) [38]. This methodology has also recently identified the IR (manuscript submitted) and Htk (EphB4) (unpublished results) as potential RTK partners for Grb14. The latter results suggest that there may be overlap in the RTK families that recruit Grb10 and Grb14.

SH2 Domain-Independent Interactions

Grb7 and Mig10 proteins contain multiple "interactive" domains that may recruit effector or regulatory molecules. The conserved N-terminal proline-rich motif represents an attractive site for recruitment of a potential effector, which would be expected to perform a fundamental role in signalling by this family. This sequence fits with the PXXP consensus for SH3-domain binding [2], and Frantz *et al.* [16] observed that the SH3 domain(s) from Abl, but not p85, Fyn or Grb2, could bind Grb10 from fibroblast cell lysates in a manner competed by this peptide. The interaction between Grb10 and Abl was not investigated in living cells, but the *in vitro* data support the hypothesis that the motif is a target for a subset of SH3 domains. Interestingly, Grb7 and Grb10 possess other proline-rich regions (Fig. 1) that may recruit member-specific effectors. Protein–protein interaction through proline-rich sequences is a property likely to be common to Mig10 proteins, although the absence of the highly conserved motif characteristic of the Grb7 family indicates that the recruitment profile is likely to differ.

Recent research on PH domains offers some clues to the function of this module and the GM region overall in Grb7 signalling. Despite low sequence identity, these domains possess a conserved overall fold, consisting of a β sandwich closed at one end by a C-terminal α helix. Furthermore, they are electrostatically polarised, with a positively charged variable region constituting a ligand-binding site [4]. A variety of ligands have been described for PH domains, indicative of the low sequence conservation of their binding surfaces. Touhara *et al.* [39] reported binding of several PH domains to βγ subunits of heterotrimeric G proteins (Gβγ), although since the interacting region extended to outside the PH module, the extent to which this represents a property of these domains *per se* is unclear. A further example of protein–protein interaction mediated by a PH domain is provided by the work of Yao *et al.* [40], who found that the PH domain of Btk bound protein kinase C. More recently, structural studies revealed that the phosphotyrosine-binding (PTB) domains of Shc and IRS-1 possess the same fold as that of PH domains [4]. Consequently, the NPXpY-based targets of these domains constitute an additional class of PH-domain ligand.

Importantly, specific phospholipids also can interact with PH domains. The PH domain of PLC-δ1 binds phosphatidylinositol (4,5) bisphosphate (PtdIns-4,5-P_2) and phosphatidylinositol (1,4,5) trisphosphate (PtdIns-1,4,5-P_3) with high affinity [41, 42], suggesting a role in both targeting to the cell membrane (and hence co-localisation with substrate) and regulation of enzyme activity. This work calls into question the physiological relevance of the relatively low affinity interaction of other PH domains with PtdIns-4,5-P_2 [43], although, in regard to the βARK PH domain, PtdIns-4,5-P_2 and Gβγ act co-operatively in effecting membrane localisation and enzyme activation [44]. Other examples of PH domain–phospholipid interaction are provided by downstream events in the PI3-kinase signalling pathway. PtdIns-3,4-P_2 binds to the PH domain of the serine/threonine kinase Akt/PKB, stimulating Akt dimerisation and hence regulating enzymatic activity [45], and PtdIns-3,4,5-P_3 binds to the PH domains in the Sec7 homology domain-containing proteins GRP1 and cytohesin-1 [46].

What are the implications of these studies in regard to the mechanism of Grb7 family signalling? The positively charged PH domain binding surface suggests that negatively charged molecules (particularly phosphorylated moieties) are likely targets, as seen with phospholipid head groups and NPXpY-containing PTB domain ligands. Because the GM region extends on both sides of the PH domain, additional sequences may act co-operatively to stabilise binding. For example, interaction of the PH domain with the membrane surface could act in concert with binding of other GM regions to integral membrane proteins or the membrane cytoskeleton to regulate subcellular localisation. Finally, investigation of whether the PH domain is likely to perform a ligand-dependent regulatory role will largely depend on identification of the effector pathway(s) coupled to the Grb7 family. Regulation of Grb7-coupled activities in response to the generation of specific second messengers may then indicate that the latter interact with the PH domain in a manner similar to that of PI3-kinase products with the PH domain of Akt.

Phosphorylation of Grb7 Family Proteins

Ooi *et al.* [11] detected a prominent mobility shift of Grb10 subsequent to epidermal growth factor (EGF), PDGF or fibroblast growth factor (FGF) stimulation that was reversible by phosphatase treatment, indicating growth factor-induced phosphorylation of a high proportion of molecules. This phosphorylation was found to be on serine residues before and after EGF administration. Similarly, Grb14 also exhibited a basal level of serine phosphorylation that was increased by PDGF treatment [12], whereas studies on Grb7 in breast cancer cells revealed phosphorylation on serine and threonine residues that was not increased after stimulation with heregulin (manuscript submitted). The results with Grb10 and Grb14 in particular suggest that a serine/threonine kinase is a regulatory, and possibly effector, component of the Grb7 family signalling complex. The identification of the kinase responsible and the sites of phosphorylation will aid the resolution of these issues.

FUNCTION OF THE Grb7 FAMILY

To date, attempts to reveal the function of the Grb7 family in mammalian cells have focussed on the role of Grb10 proteins in signalling by the IR or the IGF-IR. Two groups have identified a potential role in regulation of mitogenic signalling. O'Neill *et al.* [17] reported that microinjection of the hGrb10γ SH2 domain into fibroblasts inhibited DNA synthesis induced by insulin and IGF-I, but not by serum or EGF. One interpretation of these data is that the recombinant Grb10 SH2 domain, by blocking interaction of endogenous Grb10 with the respective receptors, inhibits an uncharacterised mitogenic signalling pathway. However, the Grb10 SH2 domain exhibits a high affinity for the juxtamembrane and activation loop tyrosine phosphorylation sites of the IR [16]. Consequently, it remains possible that the microinjected SH2 domain blocks the recruitment of IRS-1 and Shc, which bind to the juxtamembrane Tyr960 site through their PTB domains [47], or their phosphorylation by the IR kinase or both. In support of this possibility, Liu and Roth [15] found that over-expression of hGrb10β inhibited tyrosine phosphorylation of IRS-1 and recruitment of PI3-kinase to the tyrosine phosphorylated protein fraction.

Recently Morrione *et al.* [48] found that over-expression of mGrb10α inhibited IGF-I-stimulated, but not insulin, stimulated, proliferation and partly reversed IGF-IR-mediated cell transformation. Interestingly, in contrast with the results of O'Neill *et al.* [17], IGF-I-stimulated entry into S-phase was not inhibited, but the cells accumulated in the S and G_2 phases of the cell cycle. Alterations in the tyrosine phosphorylation of the IGF-IR, IRS-1 and Shc were not detected in the transfected cells, and inhibition of proliferation was not observed in cells expressing a truncated IGF-IR lacking the proposed Grb10 binding site. These data suggest that signalling through mGrb10α plays a direct role in the down-regulation of IGF-I-induced mitogenesis. However, because this isoform contains a large insertion between the proline-rich motif and the GM domain (Fig. 1), it will be important to determine whether other Grb10 proteins exhibit similar effects.

FUNCTION OF C. *elegans mig10*

The *mig10* locus was originally identified in a screen aimed at identifying genes involved in the regulation of long-range embryonic cell migration [49]. Mutants in this gene exhibit incomplete migration of four cell types including the canal-associated neurones (CANs) and hermaphrodite-specific neurones (HSNs), as well as shortening of the posterior excretory canals. Although defects in both CAN and HSN cell migrations suggested that *mig10* mutations affected the migratory mechanism itself (because the two cell types move in opposite directions), a recently published genetic mosaic analysis revealed that, at least in the development of the excretory canals, *mig10* acts cell non-autonomously [13]. This means that the *mig10* requirement is extrinsic to the motile cell and may be involved in signalling within path or target cells—for example, downstream of a cell adhesion receptor.

Currently, it is not clear how the function of Mig10 relates to that of the Grb7 family. As shown in Figure 1, although Mig10 and Grb7 family proteins exhibit a central GM domain, their overall architecture differs in that the former lack an SH2 domain but possess an additional proline-rich region at the C terminus. Additionally, the proline-rich sequences present in Mig10 proteins are not related to the conserved Grb7 family motif. It is possible that the signalling pathway linked to Mig10 and Grb7 proteins is similar and the latter family has evolved to link this pathway with RTK activation through SH2 domain interaction. Furthermore, this pathway may regulate multiple cellular responses, as seen with PI3-kinase signalling [3, 6, 45]. Alternatively, the GM domain may represent a module found in functionally distinct proteins but, owing to the high conservation of this region, studies on its mechanism and function in C. *elegans* are likely to be applicable to higher organisms.

PERSPECTIVES

The major priority for workers in this field is to identify regulatory and effector molecules that interact with Grb7 and Mig10 proteins and hence establish a signalling mechanism. Functional analyses such as those described herein for Grb10 can then be assessed in the context of the nature of these pathways and their regulation. However, competition with other signalling pathways is an aspect of Grb7 signalling that also must be taken into account. For example, it is clear that the Grb7 and Grb2 SH2 domains bind to a similar repertoire of sites; hence Grb7 expression may modulate Ras signalling. Finally, as with the elucidation of the Ras signalling pathway [7], it is likely that genetic studies in C. *elegans* will complement biochemical approaches in the determination of the mechanism and function of this enigmatic family of signalling proteins.

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Note Added in Proof

The nomenclature used in this review is that proposed by André Nantel following consultation with workers in the field. This system allows for the possibility that the same variant will be identified in different species, and should therefore be given the same isoform designation (indicated by a Greek letter). Information regarding this system is provided on the Grb7 family website (http://www.bri.nrc.ca/thomasweb/grb7.htm). However, three recent publications published since this review was submitted (Dong *et al.* [1997] *J. Biol. Chem.* **272**, 29104–29112; Dong *et al.* [1997] *Mol. Endocrinol.* **11**, 1757–1765; and He *et al.* [1998] *J. Biol. Chem.* **273**, 6860–6867) utilize a different system, in which variants are named according to the order in which they were identified. In these papers, hGrb10α corresponds to the hGrb10β isoform shown in Figure 1, hGrb10β to hGrb10γ, and hGrb10γ to hGrb10ζ (on the website).

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